

## Inhibition of apoptosis by ionomycin and zinc in peripheral blood mononuclear cells (PBMC) of leprosy patients

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### SUMMARY

PBMC from tuberculoid (BT/TT) and lepromatous leprosy (BL/LL) leprosy patients showed spontaneous apoptosis when cultured in the absence of mitogen for 24 h, which was inhibited by anti-tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) antibodies. Apoptosis was also inhibited by ionomycin and zinc, which also increased IL-2 and decreased TNF- $\alpha$  production. The increase in IL-2 production suggests a mechanism whereby dietary supplements with zinc might alter the cell-mediated immunity response in leprosy patients.

**Keywords** apoptosis leprosy peripheral blood mononuclear cells ionomycin zinc

### INTRODUCTION

Leprosy is a chronic infectious disease that affects skin and peripheral nerves. The clinical spectrum in leprosy seems to correlate with the host immune response. In tuberculoid leprosy (BT/TT), the cell-mediated response is active, Th1-type cytokines are produced (IL-2 and interferon- $\gamma$  (IFN- $\gamma$ )) and hence disease is localized. However, in lepromatous leprosy (LL/BL), there is preferential expansion of CD4<sup>+</sup> Th2 cells producing the macrophage-deactivating lymphokines IL-4 and IL-10, while the Th1-type response decreases, thereby inhibiting the production of IL-2 and IFN- $\gamma$  [1–3]. Besides these two poles, i.e. tuberculoid and lepromatous leprosy, acute inflammatory episodes called reactions also occur and they are generally acute hypersensitive reactions to bacillary antigens. These can be type I (reversal BL BT or down-grading BT BL) and type II (erythema nodosum leprosum (ENL)) characterized by fever and crops of painful red, indurated subcutaneous nodules which may result in permanent disability. The levels of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\alpha$  and IL-4 are increased in this condition.

During high bacterial load (lepromatous leprosy), the costimulatory molecules are down-regulated as well, so macrophages stimulate the cells via the T cell receptor (TCR) complex in the absence of CD28 costimulation [4]. Thus, cells cannot progress past G<sub>0</sub>/G<sub>1</sub> phase of cell cycle and become unresponsive to further stimulation, leading to T cell anergy. Recent studies have shown that anergy under various conditions can also occur due to the

defect in TCR signalling at various steps [5,6]. In order to understand the mechanism of anergy in leprosy, PBMC of leprosy patients were cultured in the presence of various mitogens (unpublished data). However, we found that cells undergo spontaneous apoptosis in culture conditions. So, in this study an attempt was made to understand the mechanism of apoptosis in leprosy patients.

### PATIENTS AND METHODS

Untreated patients visiting the leprosy clinic of Nehru Hospital attached to the Postgraduate Institute of Medical Education & Research, Chandigarh, were taken for study. Patients were classified according to the Ridley & Jopling [7] classification for leprosy. Slit skin smear and skin biopsy was carried out in all patients to confirm the diagnosis. Controls were healthy laboratory personnel and not on any type of medication.

#### *Isolation of PBMC*

Venous blood (5 ml) was withdrawn from patients in vials containing heparin after taking informed consent. The PBMC were isolated by layering the blood on Ficoll–Paque [8]. After centrifugation at 400 g for 30 min, the buffy coat was taken out, washed twice with RPMI 1640 and cells were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml benzylpenicillin, 100  $\mu$ g/ml streptomycin and containing 10% fetal calf serum (FCS). The cells were cultured in 96-well flat-bottomed culture plates in a humidified CO<sub>2</sub> chamber at 37°C.

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*Quantification of apoptosis*

Apoptosis was quantified by staining nuclei with propidium iodide (PI) and analysing fluorescence with a FACScan (Becton Dickinson, Mountain View, CA) as described by Gougeon *et al.* [9]. Briefly, following culture for 24 h, cells were collected after centrifugation at 200 *g* for 10 min. The pellet was gently suspended in 0.5 ml of hypotonic fluorochrome solution (0.1% sodium citrate with 0.1% Triton X-100) containing 20 µg per ml PI for 20 min. RNase A at a concentration of 10 mg/ml was added and cells were further incubated for 10 min at 4°C. The suspension was analysed by flow cytometry to determine PI fluorescence of individual nuclei. Apoptotic nuclei appeared as a broad hypodiploid DNA peak that was easily discriminated from the narrow peak of nuclei with normal (diploid) DNA content. Student's *t*-test was used for statistical analysis of data and *P* < 0.01 was taken as significant.

*Quantification of cytokines*

The cells were incubated either in presence or absence of ionomycin (200 ng/ml) and zinc (5 mM) for 24 h and after that monensin (2 µM final concentration) was added to cells to arrest secretion of intracellular cytokines to extracellular medium [10]. The cells were incubated for 6 h in the presence of monensin and harvested. Intracellular levels of IL-2 and TNF-α were determined from all the sets (with and without ionomycin, zinc) for each patient using flow cytometry. After culture, cells were fixed in 4% p-formaldehyde in PBS for 30 min and then incubated with PBS containing 0.1% saponin and either rabbit anti-human IL-2 or mouse monoclonal anti-human TNF-α antibody for 1 h at 37°C to allow antibodies to penetrate the cell membrane [11]. Cells were washed three times with PBS and incubated with secondary FITC-conjugated antibody for 2 h at 4°C, washed with PBS and analysed for fluorescence by FACScan. Appropriate controls (isotype-matched) were also used.

*Ca<sup>2+</sup> mobilization assay*

Lymphocytes ( $1 \times 10^6$ ) in RPMI 1640 and 1% FCS were incubated at 37°C for 20 min with fluo 3/AM (final concentration 4 µM;

Molecular Probes, Eugene, OR) and pluronic F-127 (100 µg/ml) in the dark. Cells were subsequently stimulated with anti-TCR antibody and analysed to different time lengths, and results were analysed using Chronos software.

*Western blotting*

The expression of bcl-2 in PBMC was determined by Western blotting. Equal amounts of protein were loaded on SDS-PAGE and blotted onto nitrocellulose paper. The bcl-2 protein was detected by rabbit anti-human bcl-2 antibody (Santa Cruz Biotechnology, CA) and anti-rabbit peroxidase conjugate as secondary antibody with diaminobenzidine as substrate. Densitometric scanning of the blot was done by the gel proanalyser from Media Cybernetics (IL).

*Inhibition of apoptosis*

Anti-TNF-α antibodies, anti-IL-4, IL-6, IL-1α cytokine antibodies (Amersham Life Sciences, Aylesbury, UK) at a concentration of 5 µg/ml, ionomycin (Sigma Chemical Co., St Louis, MO) at a concentration of 200 ng/ml or zinc (5 mM) were added to the cells and checked for apoptosis. The concentrations used were not toxic for normal cells as measured by apoptosis and trypan blue staining.

**RESULTS**

PBMC of leprosy patients showed spontaneous apoptosis after 24 h of culture in the absence of mitogens compared with cells from normal individuals. The percentage of apoptosis was more in lepromatous leprosy ( $n = 15$ ,  $35.68 \pm 7.09\%$  (mean  $\pm$  s.e.m.); *P* < 0.01) compared with tuberculoid leprosy ( $n = 12$ ,  $19.21 \pm 5.18\%$ ) (Table 1, Fig. 1) and was still higher in patients with reaction. Both BT/TT with type I reaction ( $n = 5$ ,  $36.54 \pm 11.6\%$ ; *P* < 0.01) and BL/LL with both type I and II reaction ( $n = 7$ ,  $51.5 \pm 16.65\%$ ; *P* < 0.001) showed significant cell death compared with control. When PBMC of these patients

**Table 1.** Percentage apoptosis in leprosy patients in the absence or presence of ionomycin and zinc

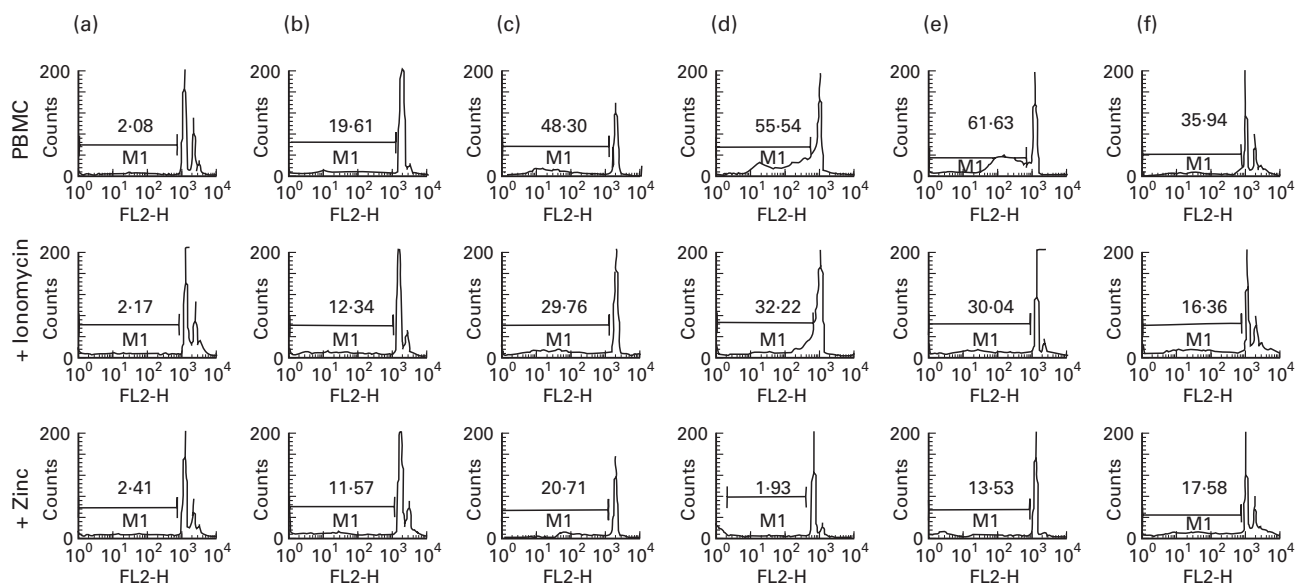
Patients	Spontaneous apoptosis	Ionomycin	Zinc
Controls $n = 8$	$7.47 \pm 1.48$	$8.0 \pm 1.24$	$7.9 \pm 0.88$
BT/TT $n = 12$	$19.21 \pm 5.18$	$10.66 \pm 4.52$	$11.56 \pm 3.5^{\dagger*}$
BT/TT in reaction $n = 5$	$36.54 \pm 11.60^{\ddagger*}$	$17.42 \pm 7.51^{\dagger*}$	$14.23 \pm 5.47^{\dagger**}$
BL/LL $n = 15$	$35.68 \pm 7.09^{\ddagger***}$	$19.55 \pm 4.46^{\dagger***}$	$16.77 \pm 3.92^{\dagger***}$
BL/LL in reaction (both type I and type II) $n = 6$	$51.5 \pm 16.65^{\dagger****}$	$27.5 \pm 7.5^{\dagger*}$	$22.75 \pm 10.2^{\dagger**}$

Values represent  $\pm$  s.e.m.

$^{\dagger}$  Versus spontaneous apoptosis of the same group.

$^{\ddagger}$  Versus control (spontaneous).

\* *P* < 0.05; \*\* *P* < 0.02; \*\*\* *P* < 0.01; \*\*\*\* *P* < 0.001.

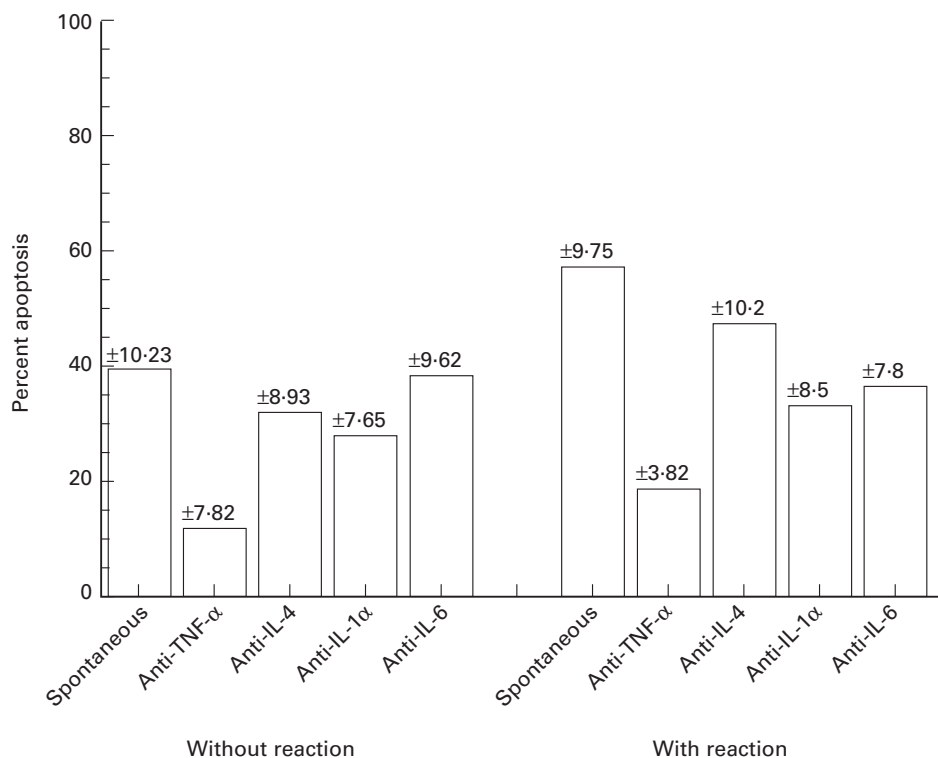


**Fig. 1.** Spontaneous apoptosis in PBMC of leprosy patients on culture for 24 h. The cells were suspended in hypotonic buffer mentioned in Patients and Methods. The cells were stained with propidium iodide and hypodiploid nuclei were detected by flow cytometry. (a) Control. (b) Tuberculoid leprosy (BT/TT). (c) BT/TT with reaction. (d) LL/BL with type II reaction. (e) LL/BL with type I reaction. (f) Lepromatous leprosy (LL/BL). Data are representative of one form each category of patients in Table 1. Area under M1 shows apoptotic population.

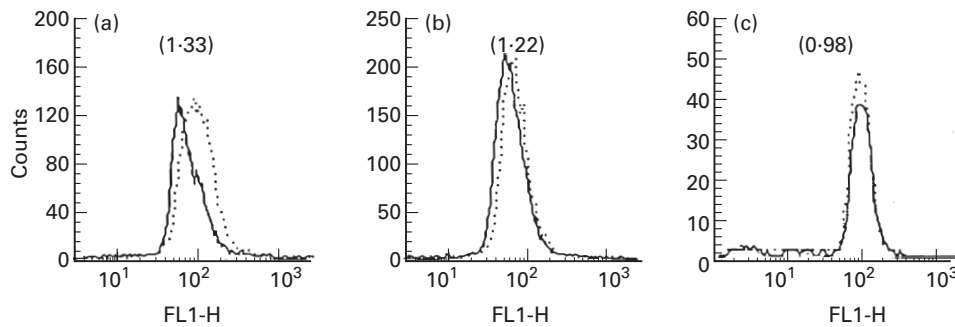
were cultured in the presence of various anti-cytokine antibodies (anti-IL-4, anti-IL-6, anti-IL-1 $\alpha$  or anti-TNF- $\alpha$  antibodies), anti-TNF- $\alpha$  antibodies could block the apoptosis in patients both with reaction ( $P < 0.001$ ) and without reaction ( $P < 0.01$ ), while anti-IL-6 or anti-IL-1 $\alpha$  inhibited apoptosis by approx. 30% only in

patients with reaction (Fig. 2), suggesting that TNF- $\alpha$  is mainly acting as an inducer of apoptosis in these cells.

When ionomycin ( $\text{Ca}^{2+}$  ionophore which facilitates entry of calcium into the cells) or zinc were added to culture (Table 1), both significantly inhibited apoptosis in patients with or without



**Fig. 2.** Effect of various anti-cytokine antibodies on inhibition of apoptosis in leprosy patients without reaction and with reaction. The antibodies were added to the wells at a concentration of 1  $\mu\text{g}$  and apoptosis was checked after 24 h. Values represent mean  $\pm$  s.e.m. ( $n = 5$ ). Five donors per group were taken for the study.



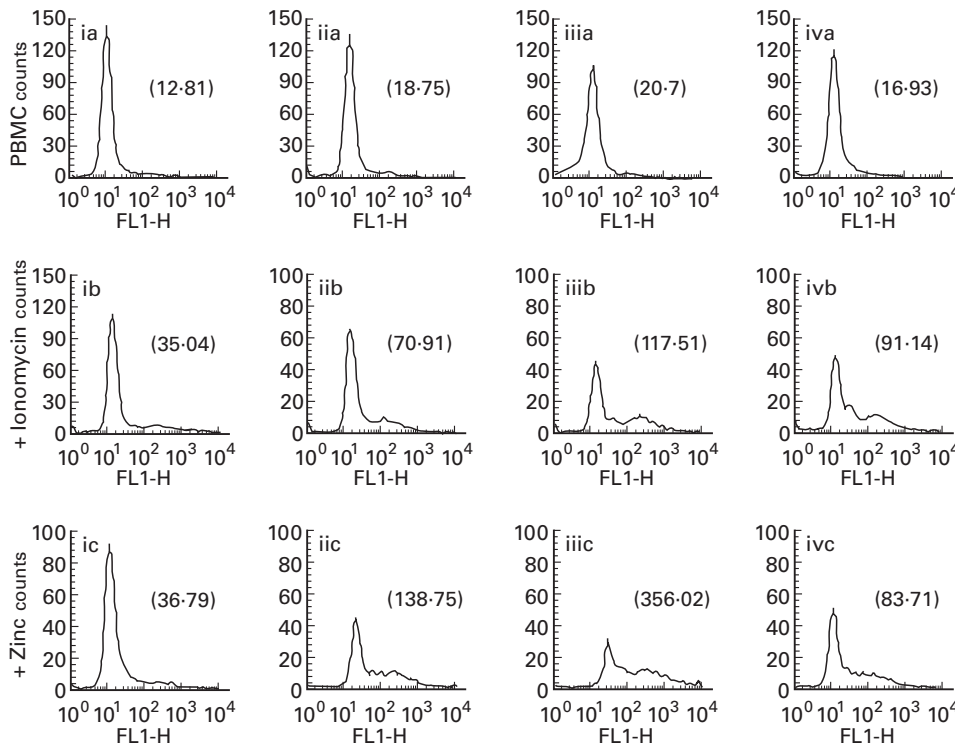
**Fig. 3.** Anti-TCR $\alpha/\beta$  MoAb-induced calcium influx in PBMC from leprosy patients. (a) Control. (b) BT/BL (type 1 reaction). (c) Lepromatous leprosy (LL). The intracellular calcium was measured by loading the cells with 4  $\mu$ M fluo 3/AM for 20 min. The cells were subsequently stimulated with anti-TCR antibody and analysed by flow cytometry. The plot shows the fluorescence intensity and values in parentheses represent mean peak-to-base ratio. Data shown are representative of one patient from each group. A total of 10 patients of each category was studied.

reaction. Ionomycin blocked apoptosis in both lepromatous leprosy ( $P < 0.01$ ) as well as in patients with reaction ( $P < 0.05$ ), whereas zinc inhibited apoptosis in all the groups, i.e. BT/TT ( $P < 0.05$ ), BL/LL ( $P < 0.01$ ) and in reaction ( $P < 0.02$ ).

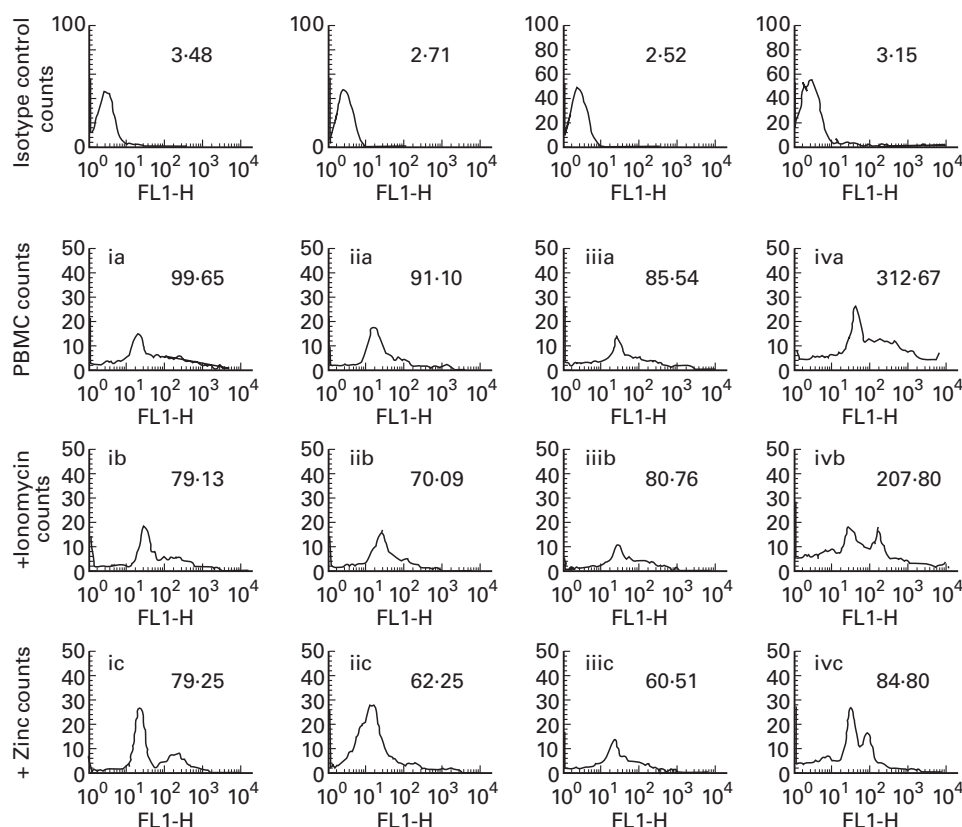
The blocking of apoptosis by ionomycin suggests that cells may be defective in calcium mobilization. To confirm this, the cells were incubated with anti-TCR $\alpha/\beta$  antibody and calcium levels were determined before and after incubation with anti-TCR $\alpha/\beta$  antibody. The results showed that anti-TCR MoAb could not significantly increase the intracellular calcium levels in lepromatous leprosy patients, whereas tuberculoid leprosy patients

showed an increase in intracellular calcium ( $P < 0.01$ ) (Fig. 3), suggesting that defective calcium mobilization in BL/LL patients may be responsible for induction of anergy and/or apoptosis in PBMC of these patients.

Since intracellular calcium levels are important in synthesis of IL-2, we also checked the expression of IL-2 in the cells using two inhibitors of apoptosis (Fig. 4). When PBMC were incubated with ionomycin (Fig. 4b) the increase in IL-2 production was almost three to four times higher in BT/TT with type I reaction (Fig. 4iiib) ( $P < 0.001$ ) when compared with control (Fig. 4iib). BL/LL patients with reaction (Fig. 4iib) and without reaction (Fig. 4ivb)



**Fig. 4.** Intracellular IL-2 production in PBMC of leprosy patients incubated in the absence (a) or presence of ionomycin (b) and zinc (c) for 24 h. (i) Healthy control. (ii) Patient with ENL (type II reaction). (iii) BT downgrading BL (type I reaction). (iv) Lepromatous leprosy (LL). Values in parentheses represent mean fluorescence intensity. The data represent FACSscan of one patient. A total of five patients of each category was studied.



**Fig. 5.** Intracellular tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels in PBMC of leprosy patients incubated in the absence (a) or presence of ionomycin (b) and zinc (c) for 24 h. After adding monensin for 5 h the cells were harvested and stained with anti-TNF- $\alpha$  antibody and subsequently with secondary FITC conjugate and analysed for fluorescence. (i) Healthy control. (ii) BT downgrading BL (type I reaction). (iii) Lepromatous leprosy (LL). (iv) Patient with ENL (type II reaction). Values in parentheses represent mean fluorescence intensity. A total of four patients of each category were studied.

did not show a significant increase in intracellular IL-2 levels when compared with control (Fig. 4ib).

Zinc was able to induce higher levels of IL-2 and the increase was approx. 8–10-fold in BT/TT (Fig. 4iic,  $P < 0.001$ ) and BL/LL patients with reaction (Fig. 4iic,  $P < 0.001$ ). In patients without reaction (Fig. 4ivc), the induction of IL-2 was not significantly enhanced when compared with control (Fig. 4ic).

The blocking of apoptosis by ionomycin and zinc in the patients with reaction also suggests that these metal ions are directly/indirectly neutralizing the effect of TNF- $\alpha$ -induced apoptosis. Moreover, ionomycin plays an important role in IL-2 synthesis and zinc is a potent immunostimulator which induces a shift from Th2 to Th1 [12]. To determine whether ionomycin and zinc can modulate the expression of IL-2 and TNF- $\alpha$  in PBMC of these patients, the levels of intracellular cytokines TNF- $\alpha$  and IL-2 were estimated.

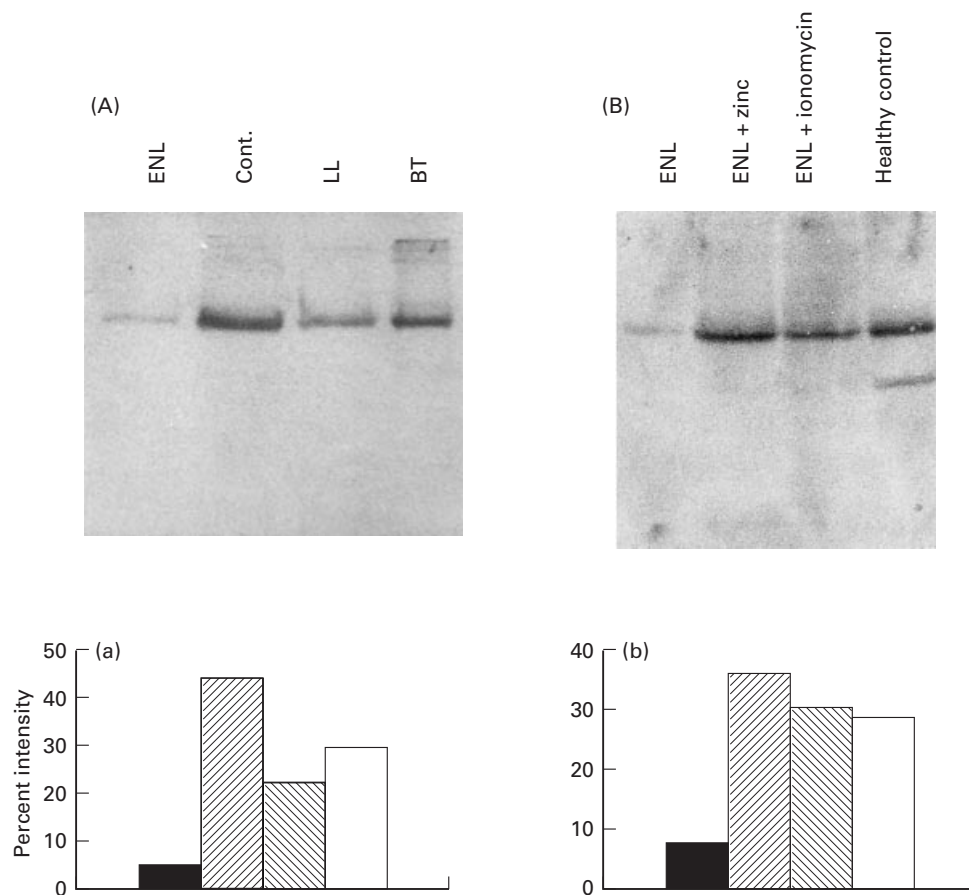
The intracellular TNF- $\alpha$  levels showed a significant decrease with zinc (Fig. 5) in the case of patients with type I reaction (Fig. 5iic,  $P < 0.05$ ) and type II reaction (Fig. 5ivc,  $P < 0.001$ ) compared with control (Fig. 5ic), whereas with ionomycin, a significant decrease was observed only in patients with type II reaction (Fig. 5ivb,  $P < 0.05$ ). No significant decrease was observed in BL/LL patients (Fig. 5iiib). Thus, the effect of ionomycin and zinc was observed only in patients with reaction with high levels of TNF- $\alpha$ .

The expression of bcl-2 (which is a major survival factor and an anti-apoptotic protein) was found to be low in PBMC of patients with a high apoptotic index (especially with type II reaction) (ENL, Fig. 6), whereas upon supplementation with ionomycin and zinc, there was a significant increase in bcl-2 levels (Fig. 6B,b) along with a simultaneous decrease in apoptosis. The bcl-2 levels were also detected by flow cytometry and similar results were obtained (data not shown).

## DISCUSSION

The pathogenesis of *Mycobacterium leprae* infection is characterized by impaired cell-mediated immunity (CMI) and increased humoral response [1–3]. When we tried to investigate the mechanism of impairment of CMI response, we found that PBMC of many patients underwent spontaneous apoptosis in culture conditions which were more in patients with reaction compared with patients without reaction.

The blocking of apoptosis with anti-TNF- $\alpha$  antibody indicated that TNF- $\alpha$  is involved in inducing cell death [13,14]. These results correlate well with the previous findings that serum levels of TNF- $\alpha$  increase with severity of disease and in both type I and type II reactions [15–18]. Moreover, it was seen that antibodies against other proinflammatory and Th2 cytokines (whose levels are also increased during leprosy infection) failed to inhibit



**Fig. 6.** Expression of anti-apoptotic protein bcl-2 in PBMC of leprosy patients. (A) Bcl-2 levels in ENL (type II reaction), control, lepromatous leprosy (LL), tuberculoid leprosy (BT) patients before incubation with ionomycin or zinc. (B) The data represent those of an ENL patient after incubation with ionomycin or zinc. (a,b) Densitometric scanning of the Western blot to quantify the expression of bcl-2. A total of seven patients of each category was studied. The experiment was repeated three times with each category of patient.

the apoptosis significantly, indicating that the effect was mainly due to  $\text{TNF-}\alpha$ .

Our previous study has already shown that cells from lepromatous leprosy patients have higher levels of basal intracellular calcium [19]. However, anti-TCR antibodies failed to increase intracellular calcium in these patients compared with control cells (Fig. 4), thereby indicating that there was a defect in calcium mobilization in PBMC of lepromatous leprosy patients. This, along with our earlier report, suggests that inability of cells to synthesize IL-2 (or the anergic state) is mainly due to proximal defect in transduction of activation signals. However, this does not rule out the possibility that downstream events of the TCR pathway are not affected by ionomycin, as ionomycin could block apoptosis by only approx. 50%. Our results are also supported by a very recent report [20] showing that Th2-type cells fail to use the calcium ion signalling pathway. Thus, an imbalance in calcium homeostasis may be responsible for apoptosis [21] and restoration of this homeostasis partially by adding ionomycin may lead to inhibition of apoptosis and synthesis of IL-2.

Zinc (known to block  $\text{Ca}^{2+}$ -dependent apoptosis by blocking calcium/ $\text{Mg}^{2+}$ -dependent endonuclease activity and an inhibitor of caspase 8) [22] was also able to block the spontaneous apoptosis significantly and was more effective in cells from ENL patients

(type II reaction). It is interesting to note that a deficiency of calcium, iron, magnesium and zinc has already been reported in serum of leprosy patients [23–26] and a non-placebo-controlled trial has shown that oral zinc therapy along with multidrug therapy in leprosy patients reduces the frequency, duration and severity of ENL reactions (type II), but its mechanism of action is not known [27,28].

An important cytokine which enhances the Th1-type response is IL-2 and its secretion is decreased in lepromatous leprosy patients, which leads to a shift towards Th2-type cytokines during progression of the disease [3]. Our results also indicate that ionomycin as well as zinc could induce IL-2 production in PBMC of these patients, which may help to overcome bacterial infections and increase the survival of cells by up-regulating the levels of bcl-2 [29]. In addition, our preliminary experiments have shown that rIL-2 also inhibits cell death in PBMC of these patients (data not shown). Moreover,  $\text{TNF-}\alpha$  (known to be an antagonist of IL-2) down-regulation may lead to an increase in intracellular IL-2 levels. Thus, our study provides the first experimental evidence of the effect of zinc and calcium ionophore on  $\text{TNF-}\alpha$  and IL-2 production.

Zinc has already been shown to induce proliferation in PBMC of HIV patients and many other diseases by inducing a shift from a Th2- to Th1-type response [12,30] and IFN- $\alpha$  levels [31], which

further supports our results that zinc might induce proliferation in anergic cells by inducing IL-2 production. Moreover, zinc is a cofactor for calcineurin (an important component of the TCR pathway) and many transcription factors, some of which activate IL-2 promoter.

Since ENL is an immunological complication in leprosy, its management demands anti-inflammatory or anti-TNF- $\alpha$  medication [19]. Many drugs are used to manage this serious complication, e.g. thalidomide, steroids [32], clofazimine, tenidap, pentoxifylline and cyclosporin A [33]. Since in our study zinc has been shown to antagonize/neutralize the effect of TNF- $\alpha$  and increase IL-2, it may offer a cheap/inexpensive therapy for treating the infection and controlling inflammation in various diseases. Further experiments are underway to determine the mechanism of action of zinc and ionomycin on inhibition of TNF- $\alpha$ -induced apoptosis of PBMC.

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